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### A Salty Compromise: The Behavioral Response of Red-Backed Salamanders (*Plethodon cinereus*) to Different Concentrations of NaCl, a Prevalent Road Deicer

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## Abstract

Many amphibians are bound to a nearby freshwater source such as lakes and vernal pools. Often times, this is because they are fully aquatic; if they are partially terrestrial, they still need freshwater bodies to lay their eggs in a suitable environment. With the constant use of road salt as a deicer during snowing periods, the subsequent runoff has negatively altered nearby soil and water sources. Amphibians, with their permeable skin, are at great risk of desiccation if exposed to a hyperosmotic environment caused by this application. However, some plethodontid salamanders deviate from this restrictive norm by living as dedicated terrestrials. Red-backed salamanders (*Plethodon cinereus*), a common plethodontid species, have the capacity to relocate to a less salty region whereas freshwater-bound amphibians are mostly confined. Few studies have examined the increased salinity on soil and the potential for terrestrial amphibians to actually react and relocate to a better setting. In this study, red-backed salamanders were placed on a surface that would vary in salinity from 0 to 0.5 M NaCl and the other with aged tap water of 0 M NaCl. As the salinity of the test side increased, salamanders behaviorally responded by spending less time there with the threshold tolerance of about 0.167 M NaCl. To examine their response to multiple regions with different salinity concentrations, they were placed in an enclosed track with 6 patches of salinity from 0 to 0.25 M NaCl for one hour. Although red-backed salamanders had a bias for the end-patches of the tracks, they were able to move from previously preferred patches to patches that contained lower salinity levels. The threshold of tolerance was 0.148 M NaCl for salamanders in this experiment; it differed from the previous 0.167 M NaCl possibly because of a smaller range among applied concentrations. This suggests that red-backed salamanders have the behavioral aptitude to determine which areas have increased salinity and avoid them when apparent. As a result, red-backed salamanders may be

able to determine appropriate sites for individual territories and persist as a population. This study provides more insight on the repercussions of road salt on terrestrial amphibians, and will aid in examining the conservation efforts needed for species that are the most at risk of this anthropogenic consequence.

MONTCLAIR STATE UNIVERSITY

A Salty Compromise: The Behavioral Response of Red-Backed Salamanders (*Plethodon cinereus*) to Different Concentrations of NaCl, a Prevalent Road Deicer

By Randolph Viola

A Master's Thesis Submitted to the Faculty of Montclair State University

In Partial Fulfillment of the Requirements for the Degree of Master of Science November 2019

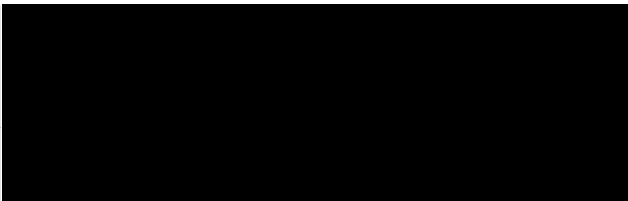
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A Salty Compromise: The Behavioral Response of Red-Backed Salamanders (*Plethodon cinereus*) to Different Concentrations of NaCl, a Prevalent Road Deicer

A THESIS

Submitted in partial fulfillment of the requirements  
For the degree of Master of Science

By  
RANDOLPH VIOLA  
Montclair State University Montclair, NJ  
January 2020

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By Randolph Thomas Viola

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## **Table of Contents**

<b>Chapter Title</b>	<b>Page</b>
Abstract	1
Thesis Signature	3
Title Page	4
Copyright Page	5
Acknowledgments	6
Table of Contents	7
List Figures and Tables	8
Introduction	9
Materials and Methods	11
Animals and Husbandry	11
Binary Choice Trials	12
Salinity Gradient Trials	13
Statistical Analysis	15
Results	16
Binary Choice Trials	16
Salinity Gradient Trials	17
Discussion	18
Figures	25
Literature Cited	39



## **List of Figures and Tables**

**Figure 1** - The mean times during binary choice trials that red-backed salamanders spent on six different concentrations of NaCl for 3600 seconds.

**Figure 2** - Red-backed salamanders and their inverse relationship between tolerance and salt concentration on a nominal logistic regression curve.

**Figure 3** - The mean amount of times that individuals crossed over to the opposite patch for each salamander morph.

**Figure 4** - The mean frequency of barrier crossings among different temperatures.

**Figure 5** - The mean number of times individuals moved to a different patch in respect to the side of test concentrations.

**Figure 6** - The mean time spent on NaCl patches for lead and red salamanders.

**Figure 7** - The mean time on test concentrations among three different temperatures.

**Figure 8** - The mean duration individuals spent on test concentrations when patches were placed on the left or right side.

**Figure 9** - The mean occurrence of patch crossings that individuals performed under each test concentration of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl.

**Figure 10** - The time red-backed salamanders spent on a patch in the control and experimental salinity gradient trials.

**Table 1** - Sequential Bonferonni technique among experimental vs. control patch comparisons.

**Figure 11** - Nominal logistic regression curve of tolerance over salt concentration during salinity gradient trials.

**Figure 12** - Salamander binary choice trial arrangement.

**Figure 13** - Salamander salinity gradient trial arrangement.

## Introduction

During snowbound periods, chemical deicers are widely used to minimize icing and assist in removing snow from roadways (Dai et al. 2012). Deicers are released in either a dried, dampened, or mixed solution state in accordance to existing road conditions. Sodium chloride is a prevalent deicer agent simply because of its low cost (Blomqvist and Johansson 1999).

Although there are organic deicers such as potassium acetate, they are rarely used as they cost approximately ten times as much as the previous option (Dai et al. 2012). While salt deicers are cheap and beneficial in keeping roads traversable, their application has lead to costly and detrimental consequences on both society and the environment (Fu et al. 2012).

Sodium chloride deicers cost a yearly estimate of around \$924/lane mile, which accumulates to a total of \$200 million/year in North America (Kelting and Laxson 2010). Taking inflation into account, these costs would be adjusted to \$1,094/lane mile/year and \$237.5 million/year. In the United States, there is a staggering \$23.4 billion (or \$27.7 billion in 2019)/year lost on deterrence, restoration, and depreciation from corrosive effects on motor vehicles alone (Koch et al. 2002). Road salts considerably increase corrosion rates on vehicles by intensifying the conductivity of trapped moisture (CASE 2015). When examining environmental value, there is a \$2,320 (or \$2,749 in 2019)/lane mile/year reduction caused by the application of road salt. These hidden deficits undermine the inexpensive expenditures of sodium chloride deicers and exemplify the destructive outcomes (Kelting and Laxson 2010). All price conversions above were calculated with the US Inflation Calculator on March of 2019. (<https://www.usinflationcalculator.com>).

Terrestrial and freshwater regions near salted roads experience altered pH levels and chemical composition (Trombulak and Frissell 2000), with high traffic speeds further promoting

this disparity (Hootman et al. 1994). There is a direct proportion of increased salinity levels in soil with respect to amount road salt applied. Road salts promote ion exchange between sodium and the calcium and magnesium ions present in the soil, resulting in the loss of necessary cations and an increased abundance of hydrogen and chloride ions within 50 m of applied sites (Lofgren 2001). Soil is also physically altered to become amorphous and dense, thereby losing overall moisture content and lowering water infiltration (TRB 1991). The excess chloride from road salt, frequently consisting of 1000 to 10,000 mg/L (Environment Canada 2001), is left to: disperse as surface runoff, infiltrate the soil surface and move along the subsurface interflow, or seep into groundwater (Bencala et al. 2018). Among the aquatic wildlife, constant exposure of more than 240 mg/L of chloride is considered destructive to around 10% and there are accounts of 4000 mg/L of chloride recorded in both ponds and wetlands nearby salted roads (Hopkins et al. 2013), and some even reaching as much as 12,463 mg/L of chloride (Ohno 1990).

Ectotherms require a considerably low amount of energy and are consequently efficient in biomass production and conversion, which provides larger organisms more access to nourishment. Their ecological contribution makes them crucial to ecosystems (Pough 1980). Even non-lethal alterations to an environment can have negative impacts on an ecosystem; if salamanders are hindered or prohibited from freely foraging, this too can negatively impact established food chains (Pough et al. 1987).

Red-backed salamanders are terrestrial plethodontids that live in forests throughout east and northeastern North America. Unlike many other amphibians, red-backed salamanders lack an aquatic larval stage. Neonates instead emerge from burrowed eggs in soil as fully terrestrial salamanders (Powell et al. 2016). As lungless salamanders, plethodontids must rely on their environment to sustain enough moisture for cutaneous respiration (Galindo et al. 2018). Any

amphibian exposed to a hypersaline environment falls under the risk of dehydration through the passive process of osmosis. Although there have been observed adaptations to prevent desiccation, saline tolerance varies among species (Shoemaker and Nagy 1977). As a purely terrestrial amphibian, there have not been many studies examining their reaction towards increased amounts of salt in the soil; most research has been towards partially or fully aquatic species.

The purpose of my study was to observe the behavioral responses that red-backed salamanders exhibited under the hyperosmotic stress of salty terrains. First, I wanted to determine if red-backed salamanders would exhibit notable aversion to salinity and spend more time on the non-saline substrate. I hypothesized that the higher the concentration of salt, the less time salamanders would occupy the saline substrate. After having a better understanding of their aversion between the control and saline patches, I observed if red-backed salamanders had the capability to avoid multiple saline patches in an enclosed setting. With closer concentrations of salt among the patches, I hypothesized that the red-backed salamanders would avoid patches that had higher saline concentrations than both the lower and non-saline patches.

## **Materials and Methods**

### Animals and husbandry:

During the fall season of 2017, Montclair State University's herpetology class and I gathered a total of 60 red-backed salamanders from the New Jersey School of Conservation in Sandyston, NJ. I collected 55 more salamanders in the same location the following fall season, which totaled 63 salamanders available for testing for the second set of experiments. We collected the salamanders from under natural cover in a forested area approximately 50 to 100 m

away from nearby roads or buildings. Salamanders were transported to Montclair State University, and were temporarily placed into plastic containers with sufficient water and air holes. Salamanders from the first year were marked for identification by injecting red or yellow visible implant elastomer tags (Northwest Marine Technology, Inc.) into either the chest or hip region. I did not inject dye into the salamanders collected in the second year, but instead identified individuals based on appearance. To ease this process, I placed all lead back morphs with two red back morphs.

Salamanders were housed in groups of three in plastic storage bins (35.6 cm x 12.0 cm x 8.9 cm) and allowed to acclimate for at least 20 weeks prior to being used in trials. I arranged each bin with moistened soil (shredded coconut bark) and a cut plastic plate shelter. I punctured holes through the lid in order to provide air circulation. Room temperature ranged from 21–24°C. Salamanders were fed appropriately-sized crickets every two to three days. If any salamander displayed unhealthy conditions such as loss of skin sheen and unresponsiveness, they were excluded from any testing.

#### Binary choice trials:

In order to determine how long and at what concentrations red-backed salamanders can tolerate road salt, I had them undergo binary choice trials to yield behavioral results. Trial arenas consisted of six 15-cm diameter petri dishes, each of which was halved by a small but traversable barrier to separate filter paper saturated with solutions of two different salinities. For each petri dish, I divided a piece of 15-cm diameter filter paper (coarse, fast filtering, and qualitative Fisherbrand P8 filter paper) into two semicircles (Figure 12). The filter paper was saturated with aged tap water (0 M) or a salt solution. One semi-circle held aged tap water, which was left in an open container for at least 2 days, for the control side of all 0 M patches. The other side held a

saline concentration of one of the following: 0, 0.1, 0.2, 0.3, 0.4, or 0.5 M NaCl. In order to avoid any possible directional bias, I rolled a die to assign the test side to either the left or right side of the petri dish. I also used this process to determine the dish allocation for each salamander of the two selected bins. If any set of two bins contained untestable salamanders, I proceeded to conduct the trial while prioritizing viable individuals into 0, 0.1, 0.2, and 0.3 M NaCl dishes; 0.4 and 0.5 M NaCl dishes were arranged as high extremes and required less analysis than the lower concentrations. No salamander underwent the binary choice trials more than once to prevent pseudoreplication.

For each trial, I used a Samsung Galaxy S5 cell phone positioned overhead as my recording device to capture salamander movement and placement. I then soaked the cut filter paper into a container with the appropriate saline solution. To avoid accidental dripping from entering the opposite side, I would gently raise and shake the soaked filter paper before placing it on the assigned side and closing the lid to avoid losing moisture. Afterwards, I began the recording and consecutively placed the six randomly assigned salamanders onto the variable side of the petri dishes. I had each salamander's trial time begin once they were dropped into the dish and ended when an hour had passed. I left the room after placing the last salamander to avoid any behavioral influence from my presence. After an hour had passed, I ended the recording and identified the salamanders. I did this after the trials to reduce the handling time before the recording session. Once I identified them, I returned the salamanders to their respective bins. I removed the filter papers and then rinsed, soaked, and rinsed again the used petri dishes with scalding tap water and dried them before proceeding to the next trial.

Salinity gradient trials:

In order to observe salinity aversion among different salt concentrations in a given area, I bought six Amerimax Home Products Dripper Flipper Downspout Extensions and cut them with shears to yield a track that was 91.4 cm long x 7.0 cm wide x 1.2 cm tall. I used 100% silicone aquarium sealant to create low barriers to partition the track into 6 15.2-cm x 7.0-cm segments (Figure 13). These patch and track dimensions not only provided suitable space for the salamanders, but the low height also minimized their ability to climb onto walls and ceilings for avoidance. The track was covered with clear vinyl, selected for its transparency and non-adhesiveness. The vinyl was secured to the edges of the track with poster putty attached to the outside edges of the tracks. The vinyl strips did not contain any holes in order to prevent any confounding variables, such as airflow, drying, and conspecific recognition. Each segment of the track was lined with medium crystalline, medium fast filtering, and qualitative filter paper (Whatman 1). The minor differences between this set of filter paper versus the previously used set would not alter results.

I implemented repeated measures for the second experiment. The first 11 trials consisted of all of the track patches containing 0 M of salinity to test for any bias towards one of the six sections. For each trial, I rolled a die to determine the location, side of entry, and salamander bin each track was assigned. I had a container full of aged tap water for all of the cut filter paper and fully submerged them. I raised and gently shook them, and quickly placed the papers into the open slots while moistening the inner walls. Whichever end patch the salamander began at was labeled as Patch 1. The adjacent patch was Patch 2, followed by Patch 3, and continued until the last patch (Patch 6). I closed the tracks with their respective vinyl ceiling after all six patches were readied and positioned them accordingly. Once placed, I began the recording and slid salamanders into their track and quickly closed it off. Similar to before, each salamander's time

trial began when they were fully inside the track. I left the room to leave the salamanders alone as well. After an hour, I ended the video and removed the salamanders from their tracks to identify and return them. I removed all pieces of filter paper from the tracks and rinsed, soaked, and rinsed the track and clear vinyl strips again with scalding water to remove any bits of substrate or mucus left behind from the salamanders. Before the next trial, I air dried all six tracks and vinyl strips before placing the new filter paper. I cleaned all tracks thoroughly with soap and hot water after completing all of the control trials.

I prepared the experimental trials similarly to the control trials; the tracks, randomized assignments, recording, and insertion method of filter papers were repeated. Instead of using exclusively aged tap water for these trials, I assigned each patch a different salt concentration in a gradient format. Every starting patch of each trial, Patch 1, contained the highest concentration at 0.25 M NaCl for this experiment; I selected this as the maximum concentration in accordance to aversion rates in the binary choice trials. Following the gradient format, Patches 2, 3, 4, 5, and 6 consisted of 0.2, 0.15, 0.1, 0.05, and 0 M NaCl respectively. I rinsed the tracks and vinyl strips with scalding water and left to air-dry after every trial.

#### Statistics:

I used JMP Pro 14.0.0 (SAS Institute Inc.) to perform statistical analyses. The significance level for ANOVAs was 0.05. I used Tukey's HSD tests to determine differences, or lack thereof, among means in which ANOVAs yielded significant results. Where multiple tests were conducted, a sequential Bonferroni correction of the P-values was applied (Rice 1989).



## Results

### Binary choice trials:

Red-backed salamanders spent more time on test patches of 0 and 0.1 M NaCl than test patches of higher salinity (Figure 1; ANOVA:  $F_{5,44} = 15.6095$ ,  $P < 0.0001$ ,  $N_{0-0.5} = 10, 9, 10, 10, 7$ , and 4). The time spent on 0 M NaCl was significantly greater than the time spent on 0.2, 0.3, 0.4, and 0.5 M NaCl. Time spent on 0.1 M NaCl, although lower than 0 M NaCl, was also significantly greater than the time spent on 0.3, 0.4, and 0.5 M NaCl (Figure 1; Tukey's HSD tests).

Time spent on the test vs. control side during a trial was converted to a binary aversion/tolerance variable. To establish whether salamanders were showing tolerance or aversion to a saline patch, I averaged the total time spent on test/starting sides of the 0 M NaCl vs. 0 M NaCl control trials and halved it. This would account for extreme behavioral variations that do not represent tolerance to a test side, such as excessive pacing and inactivity. I used the resulting 830 seconds as the determinate baseline for the nominal logistic regression analysis (Figure 2). If salamanders spent more than 830 seconds on a saline patch, then they showed tolerance; any lower amount of time spent on the patch was deemed as showing aversion. The nominal logistic regression analysis presented an inverse relationship between tolerance and salt concentration; as salt concentration increased, tolerance displayed by salamanders decreased ( $\chi^2 = 34.806$ ,  $P < 0.0001$ ). The  $EC_{50}$  value, which was the salt concentration at which 50% of salamanders began displaying aversion, was 0.167 M NaCl (95% C.I. = 0.108-0.224).

There was no significant difference between the color morph of the salamanders and the number of times traversed to a different patch (Figure 3; ANOVA:  $F_{1,48} = 1.2041$ ,  $P = 0.2780$ ,  $N_{red} = 40$ ,  $N_{lead} = 10$ ). Temperature had no significant effect on the number of times a salamander

crossed over the barrier (Figure 4; ANOVA:  $F_{1,48} = 0.0521$ ,  $P = 0.824$ ). The substrate location did not influence a salamander's activity (Figure 5; ANOVA:  $F_{1,48} = 0.3666$ ,  $P = 0.5477$ ).

The color morph did not impact the time individuals spent on test sides (Figure 6; ANOVA:  $F_{1,48} = 0.0854$ ,  $P = 0.7713$ ,  $N_{\text{red}} = 40$ ,  $N_{\text{lead}} = 10$ ). Temperature had no effect on the time spent on test concentration (Figure 7; ANOVA:  $F_{1,48} = 0.1246$ ,  $P = 0.7257$ ). Placement of the test concentration on either the left or right side had no significant influence on the time spent on saline patches (Figure 8; ANOVA:  $F_{1,48} = 0.0158$ ,  $P = 0.9006$ ). Activity was significantly lower on trials that contained test concentrations of 0.3 and 0.4 M NaCl than 0 M NaCl (Figure 9; ANOVA:  $F_{5,44} = 3.7660$ ,  $P = 0.0063$ , Tukey's HSD test).

#### Salinity gradient trials:

In the control trials, red-backed salamanders spent more time on Patches 1 and 6 (the ends of the track) than Patches 2, 3, 4, and 5 (Figure 10; ANOVA:  $F_{5,306} = 43.4779$ ,  $P < 0.0001$ ). Time spent on Patch 1 was significantly greater than all of the other patches, and time spent on Patch 6 was significantly greater than time spent on Patches 2, 3, 4, and 5 (Figure 10; Tukey's HSD tests). In the experimental trials, salamanders spent more time on Patches 5 and 6 than Patches 1, 2, 3, and 4 (Figure 10; ANOVA:  $F_{5,306} = 32.3663$ ,  $P < 0.0001$ ). Salamanders spent significantly more time on Patch 6 than every other patch, and significantly more time on Patch 5 than Patches 1, 2, 3, and 4 (Figure 10; Tukey's HSD tests).

When comparing the time spent between control and experimental trials per individual patch, individual salamander ID was used as a random effect in the statistical model to account for repeated measures. ID accounted for 0, 21.798, 17.624, 6.390, 0, and 19.861% of the variation in the model for Patches 1, 2, 3, 4, 5, and 6. A Student's t-test could determine if there was a significant difference at each concentration, but because all six concentrations were

simultaneously situated within each trial, I used the sequential Bonferroni technique (Rice 1989) on fixed effect  $P$  values to prevent the likelihood of a type-I error from occurring (Table 1). At a significance level of 0.05 ( $\alpha$ ), I calculated an adjusted initial significance level in respect to the number of tests ( $k$ ) in the analysis ( $\alpha/k = 0.0083$ ). As the lowest probability value of the six comparisons (Patch 1  $P = 1.275 \times 10^{-9}$ ) was less than the adjusted initial significance level, there was a significant difference in the analysis overall. The remaining patches were compared to another adjusted significance level ( $\alpha/(k - 1) = 0.01$ ). Patch 2 ( $P = 6.995 \times 10^{-7}$ ), Patch 6 ( $P = 0.0001$ ), and Patch 5 ( $P = 0.0002$ ) had a significant difference between the control and experimental times spent; there were no significant differences in Patches 4 ( $P = 0.0716$ ) and 3 ( $P = 0.1010$ ). Individual salamanders had no influence in the amount of time spent in a patch.

Similar to the previous experiment, I converted the time spent on each experimental patch into a binary variable of aversion or tolerance for a nominal logistic regression (Figure 11). Every control patch's mean time spent was halved and established as the threshold for that particular patch; any experimental mean time above the baseline was shown as tolerance, and any mean lower was shown as aversion. The threshold times for Patches 1, 2, 3, 4, 5, and 6 were 810, 163, 133, 125, 123, and 446 seconds. When defining each experimental patch as a concentration of NaCl, tolerance decreased as salinity concentration increased ( $\chi^2 = 104.4962$ ,  $P < 0.0001$ ). The concentration in which 50% of salamanders started showing aversion ( $EC_{50}$ ) was 0.148 M NaCl (95% C.I. = 0.131-0.166).

## Discussion

Red-backed salamanders spent the most time on experimental patches that contained the lowest salt concentrations of 0 and 0.1 M NaCl. From 0.2 M NaCl onward, salamanders deemed the saline patches unfavorable in comparison to salt-free patches and thus spent significantly less

time on them (Figure 1). While there was no significant difference between 0.5 M and 0.2 M NaCl, this could most likely be caused by the low sample size of 0.5 M NaCl trials ( $N = 4$ ). Such a high concentration is assumed to be the equivalent of seawater in lab settings (Galama et al. 2014). As some salamanders were debilitated and unable to be tested, I prioritized the testable salamanders on the lower concentrations over the higher-end concentrations. Concentrations of 0.5 M NaCl and, to a certain extent, 0.4 M NaCl were set up as my intolerable extremes, and this is backed as all times recorded in these concentrations were considered below the tolerance time requirement of 830 seconds and significantly higher than the  $EC_{50}$  salt concentration of 0.167 M (Figure 2).

The concentration of the test patch did have an influence on how often salamanders traversed between the patches (Figure 9). Salamanders that were placed in petri dishes with only 0 M NaCl patches moved more than those that were placed in the presence of 0.3 and 0.4 M NaCl, but not 0.5 M NaCl. Similar to the time comparisons above, movement of salamanders in 0.5 M NaCl might not be accurately represented as there were only 4 salamanders tested under that concentration. The ambient temperature of the testing lab had no influence on salamander activity (Figure 4), nor did the color morph of the salamanders (Figure 3). Red-backed salamanders can either be the red-striped morph or the lead-phase morph in accordance to the climate of the environment (Gibbs and Karraker 2006), and there are some behavioral differences exhibited by them. When under the threat of predation, red-striped morphs are less likely to move around as a response than lead-phase morphs (Venesky and Anthony 2007). While this study did place these salamanders in hostile scenarios, it seems that these threatened movement patterns did not translate from anti-predatory into anti-location responses. Perhaps red-backed salamanders moved around more at 0 M NaCl than 0.3 and 0.4 M NaCl as they were

free to move around without the influence of unpleasant conditions. For salamanders that were placed on saline patches, they might have needed to reside more on the aged tap water/0 M NaCl patches to rehydrate from water lost by beginning on the saline side (Heatwole 1962).

With the salinity gradient control trials, red-backed salamanders had a clear preference for the end patches of the track, especially for the end they started on (Figure 10). This track-end preference could be because the ends had a wall that provided additional security for them. For the initial patch preference, salamanders were possibly content with that patch and chose to minimize or even decline exploration. If they moved, they tended to move all the way to the other end of the track. However, this partiality was lost during the experimental trials in favor of gravitating towards less salty patches (Figure 10). The furthest patch was the most favored on account of the bias noted earlier and that it had a concentration of 0 M NaCl. What was noteworthy was that the time difference of the second furthest patch of 0.05 M NaCl; it garnered the second highest amount of time spent among the salamanders despite being among the least preferred spots on the control trials. According to both the binary choice and salinity gradient  $EC_{50}$  values of 0.167 M NaCl (Figure 2) and 0.148 M NaCl (Figure 11), a concentration of 0.05 M NaCl is considered a tolerant, if not isosmotic, concentration for red-backed salamanders. Thus, they would prefer to stay at a lower salt concentration even if that results in compromising their security. This would likely be in order to prevent immediate dehydration from exposure to their hyperosmotic surroundings. While my experiments do support behavioral avoidance of salinity within a short time span, long-term effects such as chronic exposure and possible acclimation are still unexplored.

Because red-backed salamanders lack any tolerance of freezing temperatures, they instead practice behavioral avoidance during subzero conditions and relocate (Storey and Storey

1986). They will attempt to burrow deep enough in the soil to remain active underground during winter seasons (Woolbright and Martin 2014). Thus, deeply burrowed individuals have the capacity to avoid runoff road salt from nearby streets. Runoff can afflict nearby water sources with concentrations from 1000 (Environment Canada 2001) to as high as 12,463 mg/L Cl (Ohno 1990), and much of it begins by first penetrating and seeping down into the soil (Bencala et al. 2018). These concentrations would be the equivalent of about 0.03 to 0.35 M NaCl. According to my results, salamanders should be able to react to harmful salt concentrations, thereby allowing them to relocate elsewhere while underground as well as on the surface. Subsurface movement during freezing weather sessions is only applicable, however, if they are burrowed deep enough to remain active; they would be at the mercy of their chosen location otherwise.

Although there have been numerous studies about saline tolerance of many amphibians, there are no studies that specifically examine the saline tolerance of red-backed salamanders. Many of these studies also examined salt concentrations in vernal pools or lakes rather than soil; most amphibians must find a body of water to reproduce and these locations serve as central hubs for many studies. If one is to compare salinity tolerance among different species, then they must account for the distinguishing methodology as well. It becomes far more difficult to reliably compare findings that differ in both species and methodology (Hua and Pierce 2013). The closest comparison to my findings would possibly be another plethodontid group. *Batrachoseps*. *B. attenuatus* and *major* are terrestrial salamanders found along the west coast of the United States, and for the purpose of their study, Jones and Hillman (1978) found no physiological differences between the species and grouped them together. They concluded that both species were able to tolerate a chronic setting of 0.25 M NaCl without any deaths for several days. When tested on the higher concentrations of 0.325 to 0.4 M NaCl, several

salamander deaths occurred. However, their osmotic analysis suggests the possibility of long-term tolerance to the aforementioned concentrations if given an appropriate time to acclimate. While this does not directly apply to salinity tolerance in red-backed salamanders, it does infer the possibility of evolutionary adaptation to combat osmotic water loss within the family Plethodontidae.

Plethodontids can be regarded as a reliable ecological indicator for presenting environmental concerns because of, but not limited to, their abundance, ease of experimentation, sensitivity, and trophic importance (Welsh and Droege 2001). My findings support that red-backed salamanders do exhibit appropriate aversion to hyperosmotic stress from road salting, and the death or relocation of populations could possibly lead to negative alterations on an ecosystem scale. However, this study only examined one population that resided away from high traffic roads and may not accurately represent the species. Perhaps salinity tolerance is dependent on the population's distance from roadways and level of salt exposure, and phenotypic plasticity or local adaptation occurred as a response. In addition to examining long-term effects, future studies can study different populations and examine whether salinity tolerance varies among them. If researchers do find significant variability among the populations, then they could examine whether the tolerance comes from phenotypic plasticity or local adaptation by developing and raising the offspring of examined populations in a controlled lab setting (Gao et al. 2018).

Perhaps the quickest method to lower deicer usage is to incorporate improved technology. When utilizing advanced weather prediction programs, there is more awareness of where and when to deploy plowing vehicles, and effectively aids in determining how many pounds of deicers would be required to keep the roads open. This would also provide a net

decrease in cost when compared to the status quo (Ye et al. 2009). While this does control usage and application, this still does not stop the usage of chloride deicers.  $\text{CaCl}_2$  is sometimes chosen instead of  $\text{NaCl}$  because of its efficacy under colder temperatures. However, its cost has made its usage limited to severe conditions (USEPA 2002).  $\text{MgCl}_2$ , and to a lesser extent,  $\text{CaCl}_2$  causes more damage to concrete infrastructure than its sodium counterpart (Shi et al. 2009). Acetate-based deicers have also proven to deteriorate the cement component of concrete at faster rates. In fact,  $\text{NaCl}$  has been the least destructive towards concrete roads than every other chloride and acetate deicer (Lee et al. 2000).

In terms of environmental impacts, more research is necessary on alternative deicers and their long-term consequences (Ramakrishna and Viraraghavan 2005). Something to note is that some of the alternative chloride deicers are composed with the molecular arrangement of two chloride anions per metal cation. If there were solutions of  $\text{NaCl}$  and  $\text{CaCl}_2$  that contained the same molarity, the resulting osmotic concentration from disassociated  $\text{CaCl}_2$  anions would always be doubled that of  $\text{NaCl}$  anions and thereby further increasing the osmotic pressure. It remains to be seen if this difference in osmotic pressure involved in the root of deviation between the chloride deicers.

What could possibly be the most prospective alternative for both fiscal and environmental demands is a beet juice-based deicer. Beet juice provides enough adhesiveness for road salts to lower application on roads and displacement off roads, as well as improve the efficacy of  $\text{NaCl}$  at lower temperatures. Some commercially available beet juice derivatives are obtainable as a common by-product of alcohol distilleries, stimulating usage in the Midwest (USEPA 2009). This could potentially save millions of dollars in corrosive damage from untreated road salts. However, the presence of organic additives like beet juice has been found to affect nearby



freshwater pools and promote algal growth, limiting the abundance of dissolved oxygen for aquatic organisms (Schuler et al. 2017). Mosquitos seem to benefit from the increase in organic additives by deducting a day out of their standard 15-day emergence cycle. The decrease in dissolved oxygen may lower their risk from predators as well, but the impacts of early emergence towards their fecundity needs to be further investigated (Schuler and Relyea 2018). Unfortunately, many of these alternatives are advertised as eco-friendly products when there is not enough research or evidence to back these claims (Natile and Solan 2019). Beet juice-chloride mixes seems to have the most potential when reducing road salt runoff, but more studies should be done on it and other alternatives before advocating a widespread shift in road management. For instance, the fragrance of beet juice may prompt animals to approach these roads, putting them and motorists at risk of injury. Perhaps a viable first step would be to examine how generalists or indicator species, such as American bullfrogs or red-backed salamanders, react to these alternatives and then proceed to delve into more delicate species.

## Figures

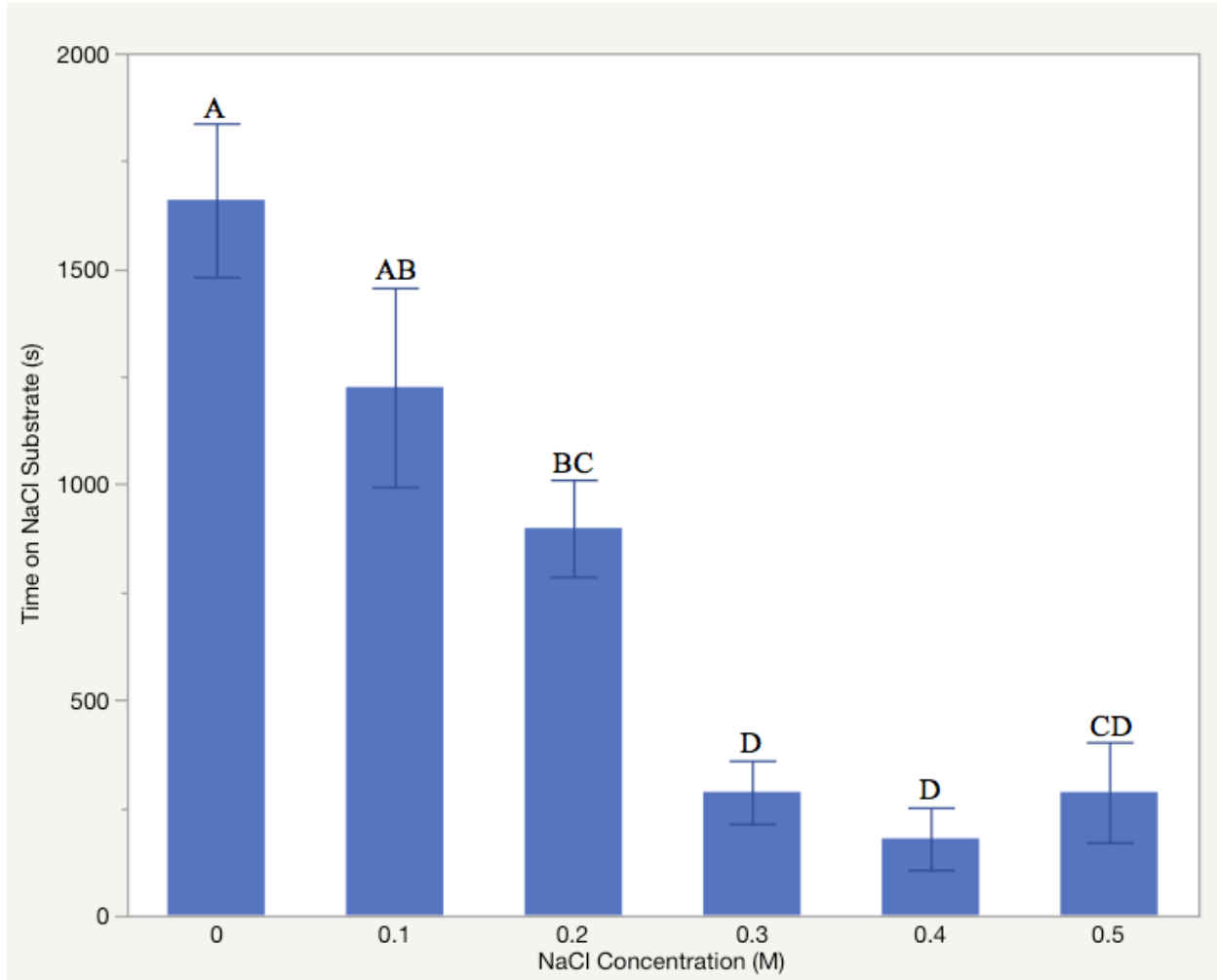


Figure 1. Salinity tolerance of red-backed salamanders during binary choice trials. The mean times during binary choice trials (mean  $\pm$  standard error) that red-backed salamanders spent on six different concentrations of NaCl for 3600 seconds. ANOVA:  $F_{5,44} = 15.6095$ ,  $P < 0.0001$ . Mean concentrations are significantly different if they do not share similar letters (Tukey's HSD tests).  $N_{0.05} = 10, 9, 10, 10, 7$ , and 4.

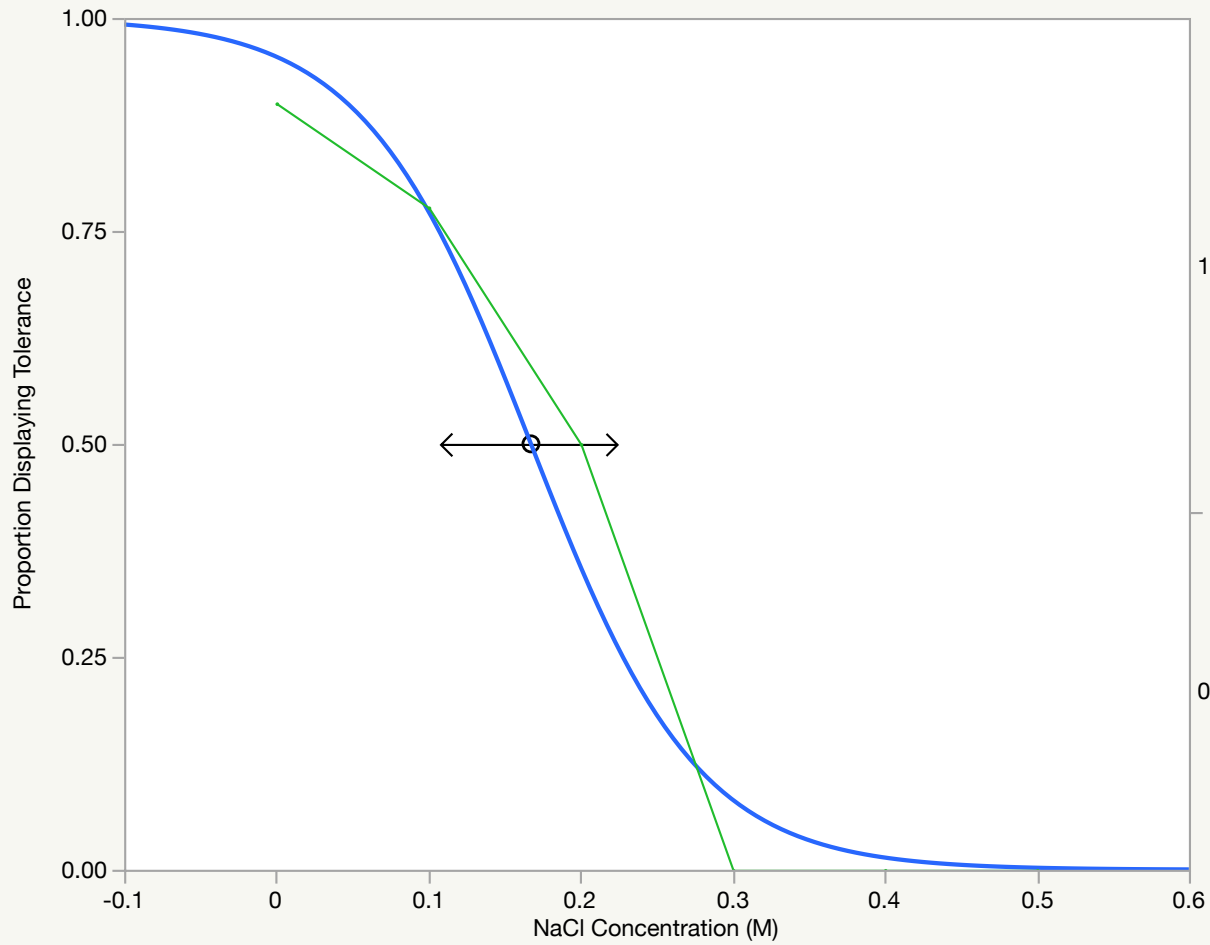


Figure 2. Salinity tolerance of red-backed salamanders during binary choice trials. Red-backed salamanders and their inverse relationship between tolerance and salt concentration on a nominal logistic regression curve ( $\chi^2 = 34.806$ ,  $P < 0.0001$ ).  $EC_{50} = 0.167$  M NaCl (95% C.I. = 0.108-0.224). A salamander was defined as showing tolerance if it spent more than 830 seconds in the test solution.

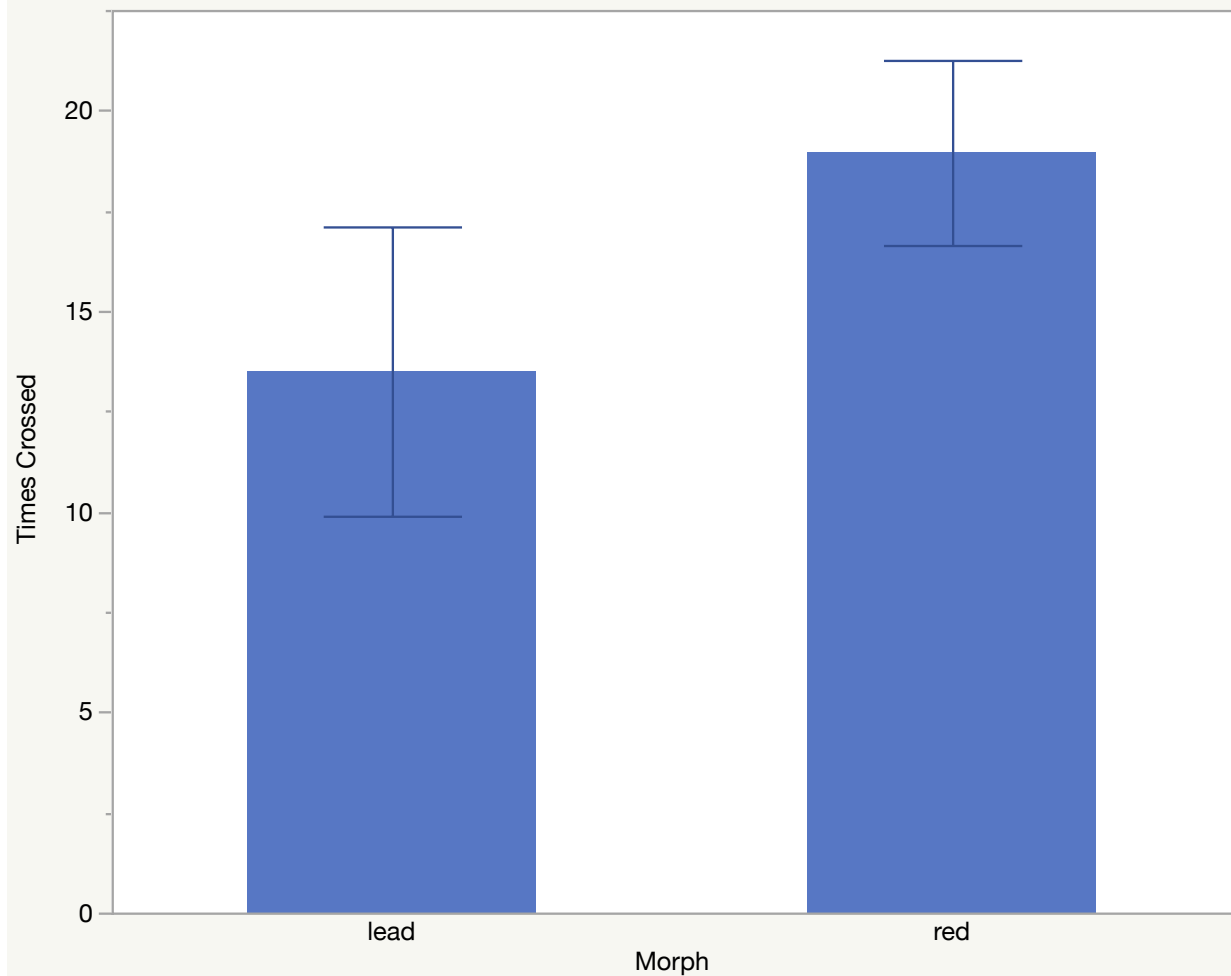


Figure 3. Activity of salamanders in accordance to color morph in binary choice trials. The mean amount of times that individuals crossed over to the opposite patch for each salamander morph (mean  $\pm$  standard error). ANOVA:  $F_{1,48} = 1.2041$ ,  $P = 0.2780$ ,  $N_{\text{lead}} = 10$ ,  $N_{\text{red}} = 40$ . There was no significant difference in times crossed between the two morphs.

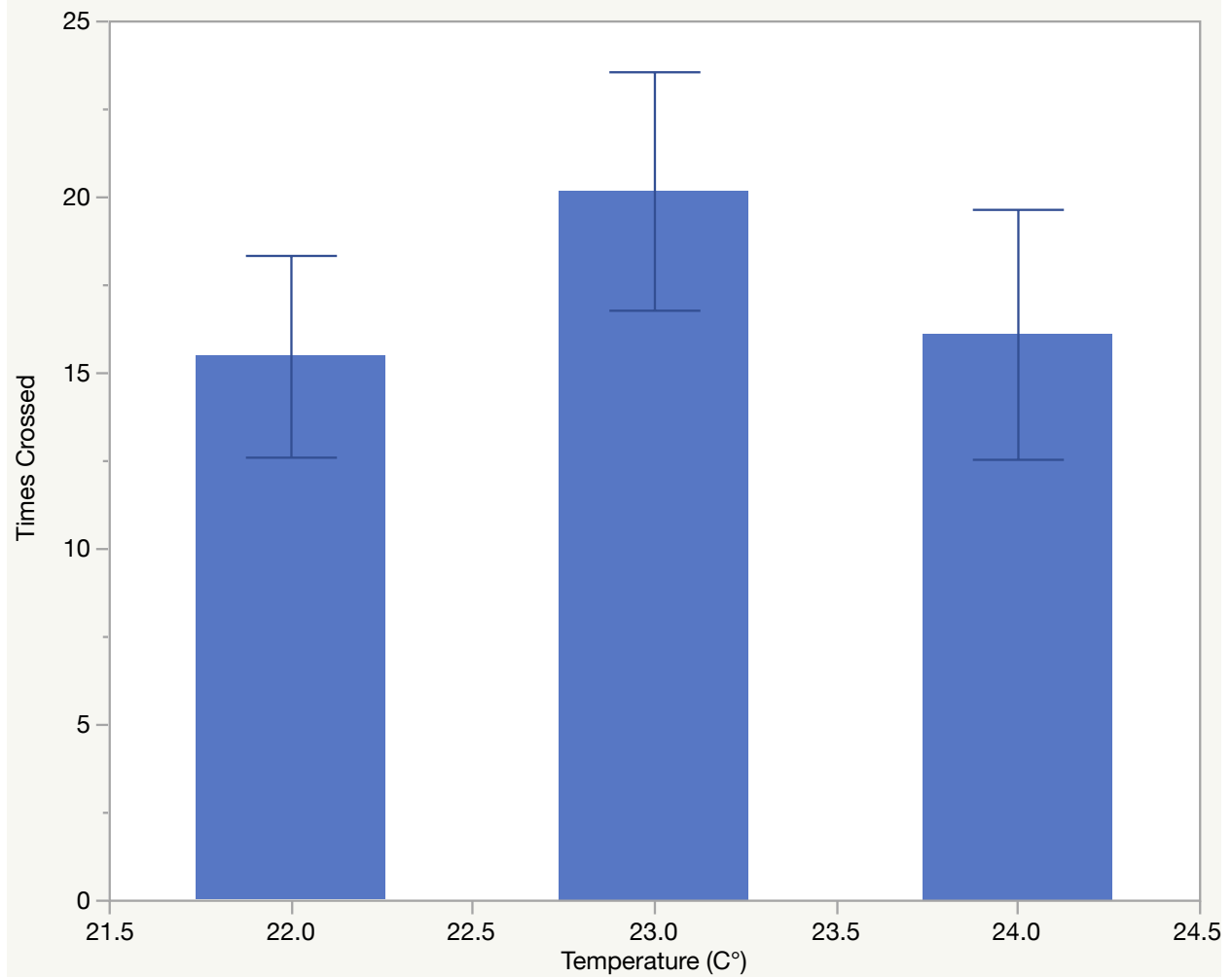


Figure 4. Activity of salamanders under the three ambient temperatures during binary choice trials. The mean frequency of barrier crossings among different temperatures (mean  $\pm$  standard error). ANOVA:  $F_{1,48} = 0.0521$ ,  $P = 0.824$ . There was no significant difference on crossing activity from temperature.

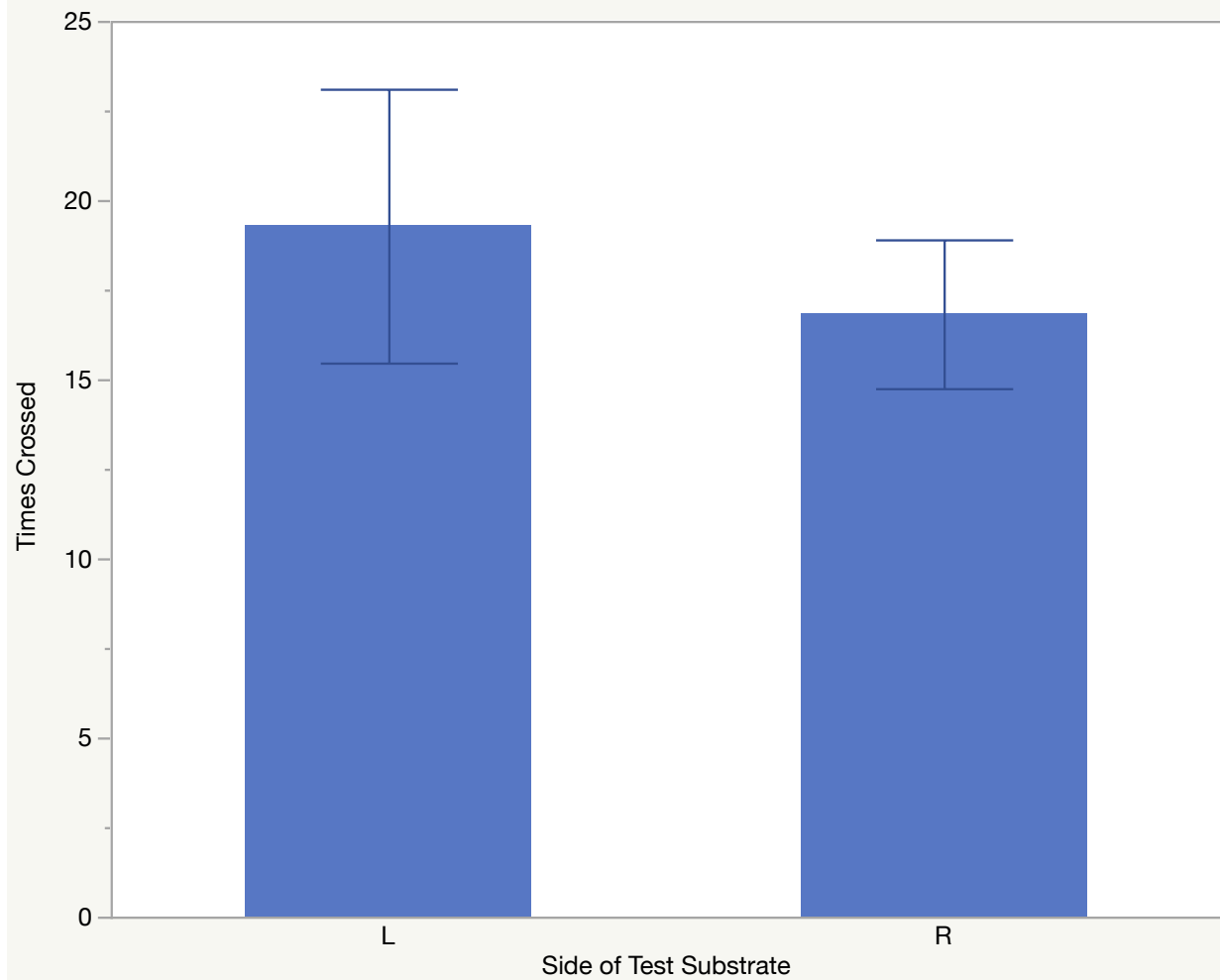


Figure 5. Activity of salamanders on each test side during binary choice trials. The mean number of times individuals moved to a different patch in respect to the side of test concentrations (mean  $\pm$  standard error). ANOVA:  $F_{1,48} = 0.3666$ ,  $P = 0.5477$ . There was no significant difference on crossing activity from test side assortment.

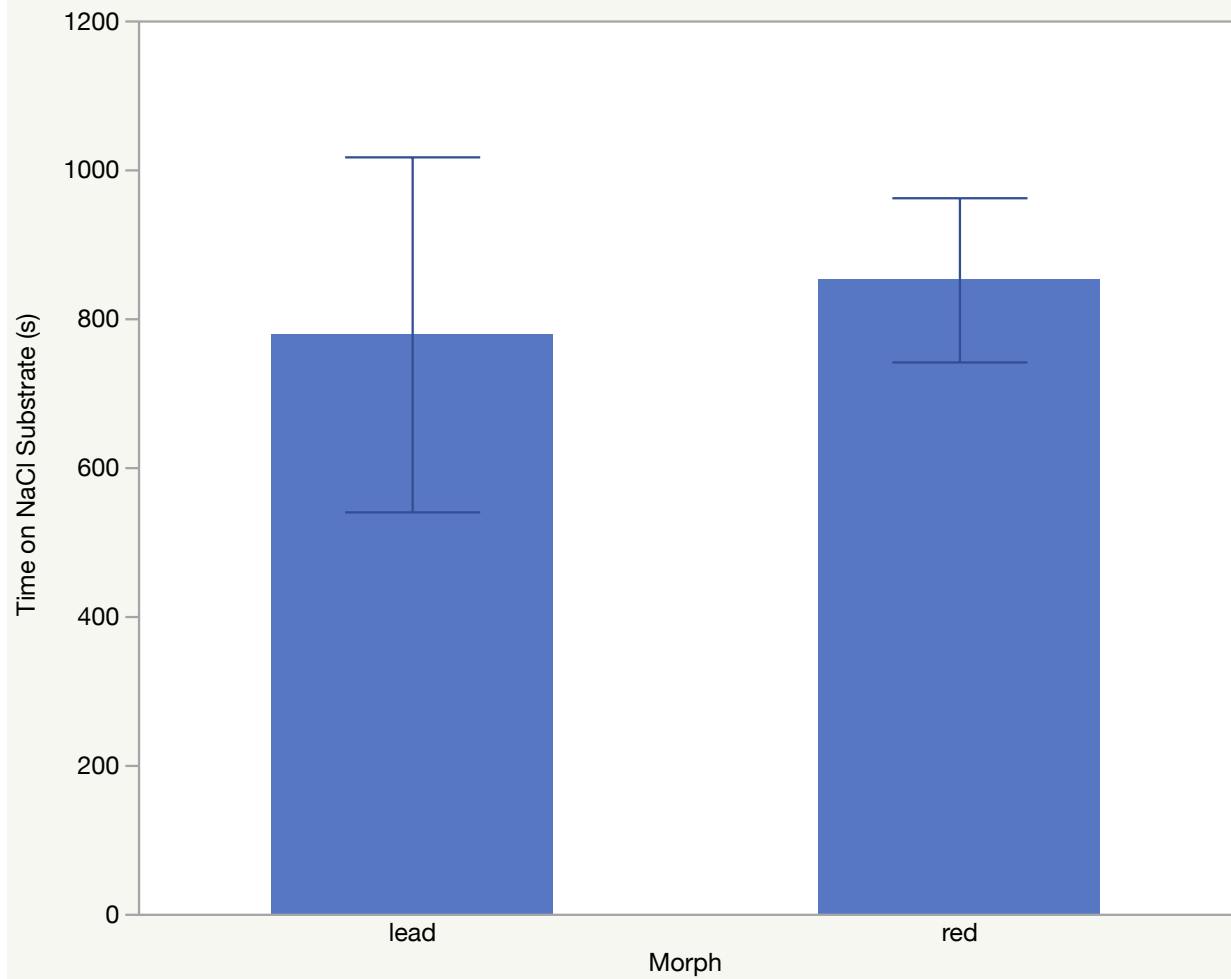


Figure 6. Salinity tolerance salamanders exhibited in binary choice trials for each color morph.

The mean time spent on NaCl patches for lead and red salamanders (mean  $\pm$  standard error).

ANOVA:  $F_{1,48} = 0.0854$ ,  $P = 0.7713$ ,  $N_{\text{red}} = 40$ ,  $N_{\text{lead}} = 10$ . There was no significant difference

between lead and red morphs on saline tolerance.

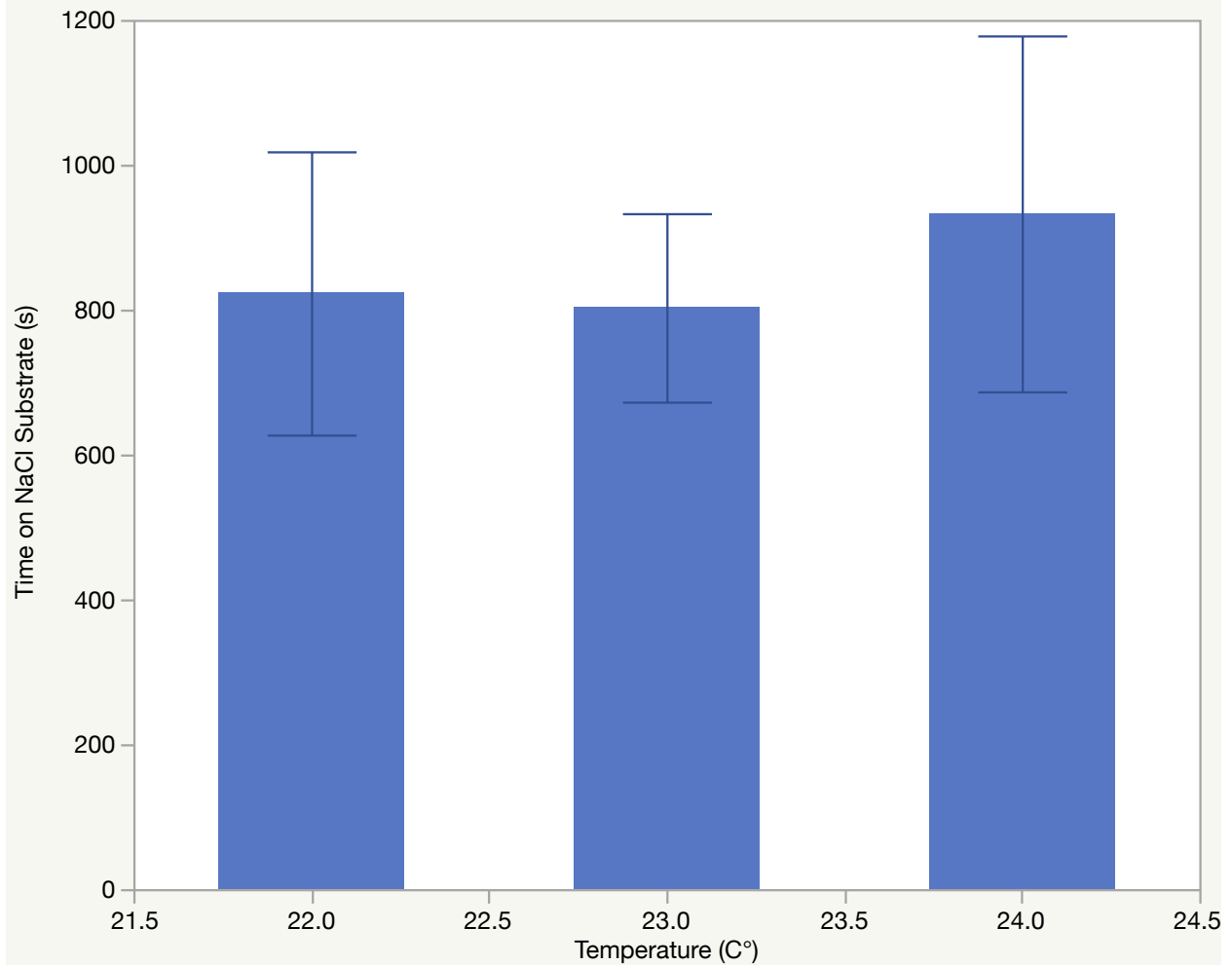


Figure 7. Salinity tolerance of salamanders among ambient temperatures recorded under binary choice trials. The mean time on test concentrations among three different temperatures (mean  $\pm$  standard error). ANOVA:  $F_{1,48} = 0.1246$ ,  $P = 0.7257$ . There was no significant difference on the amount of time spent on a test concentration in regard to trial temperatures.



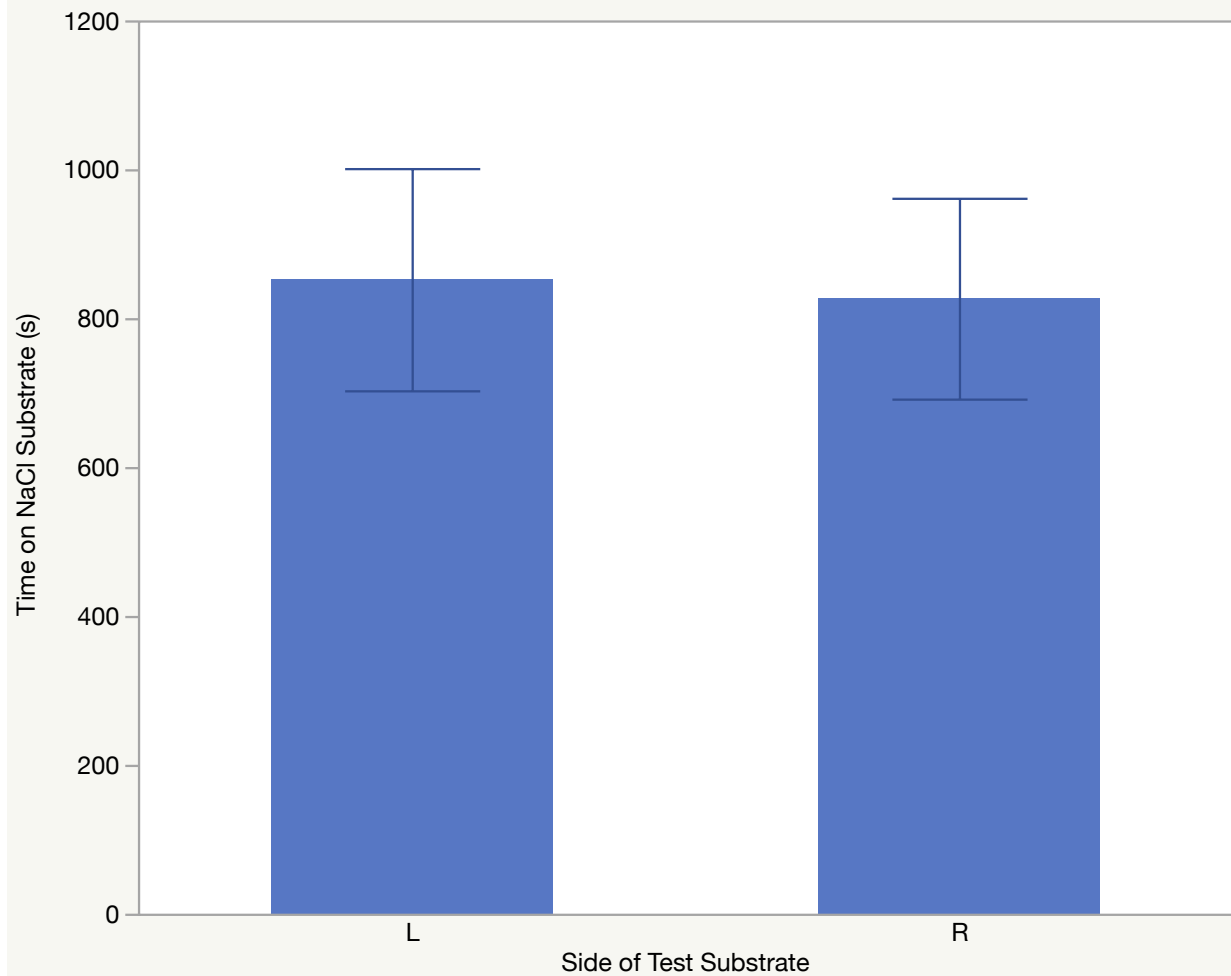


Figure 8. Salinity tolerance of salamanders on each test side during binary choice trials. The mean duration individuals spent on test concentrations when patches were placed on the left or right side (mean  $\pm$  standard error). ANOVA:  $F_{1,48} = 0.0158$ ,  $P = 0.9006$ . There was no significant difference of time spent on test patches by side placement.

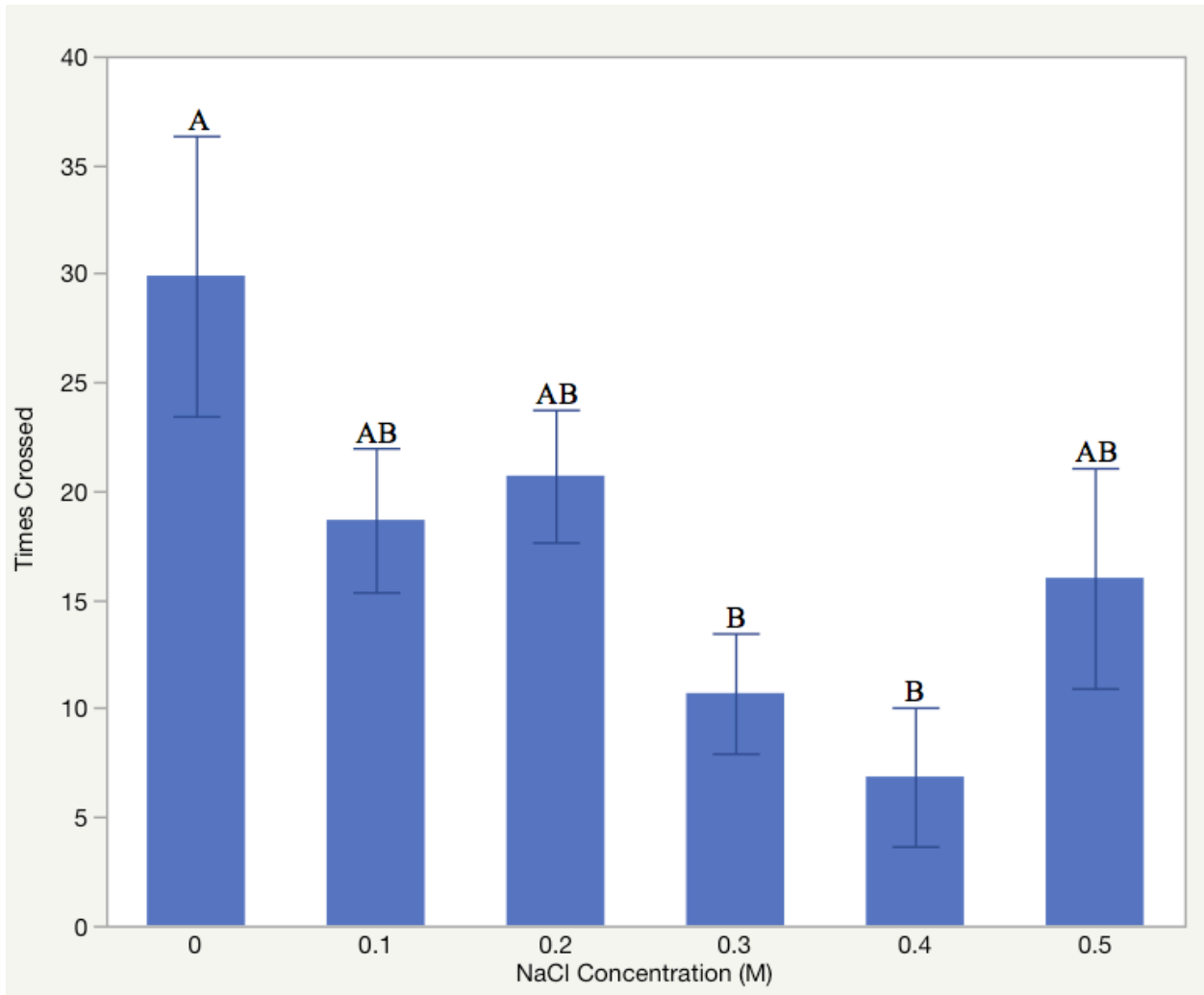


Figure 9. Activity of red-backed salamanders under each test concentration during binary choice trials. The mean occurrence of patch crossings that individuals performed under each test concentration of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl (mean  $\pm$  standard error). ANOVA:  $F_{5,44} = 3.7660$ ,  $P = 0.0063$ . Mean crossings are significantly different if they do not share similar letters (Tukey's HSD tests).

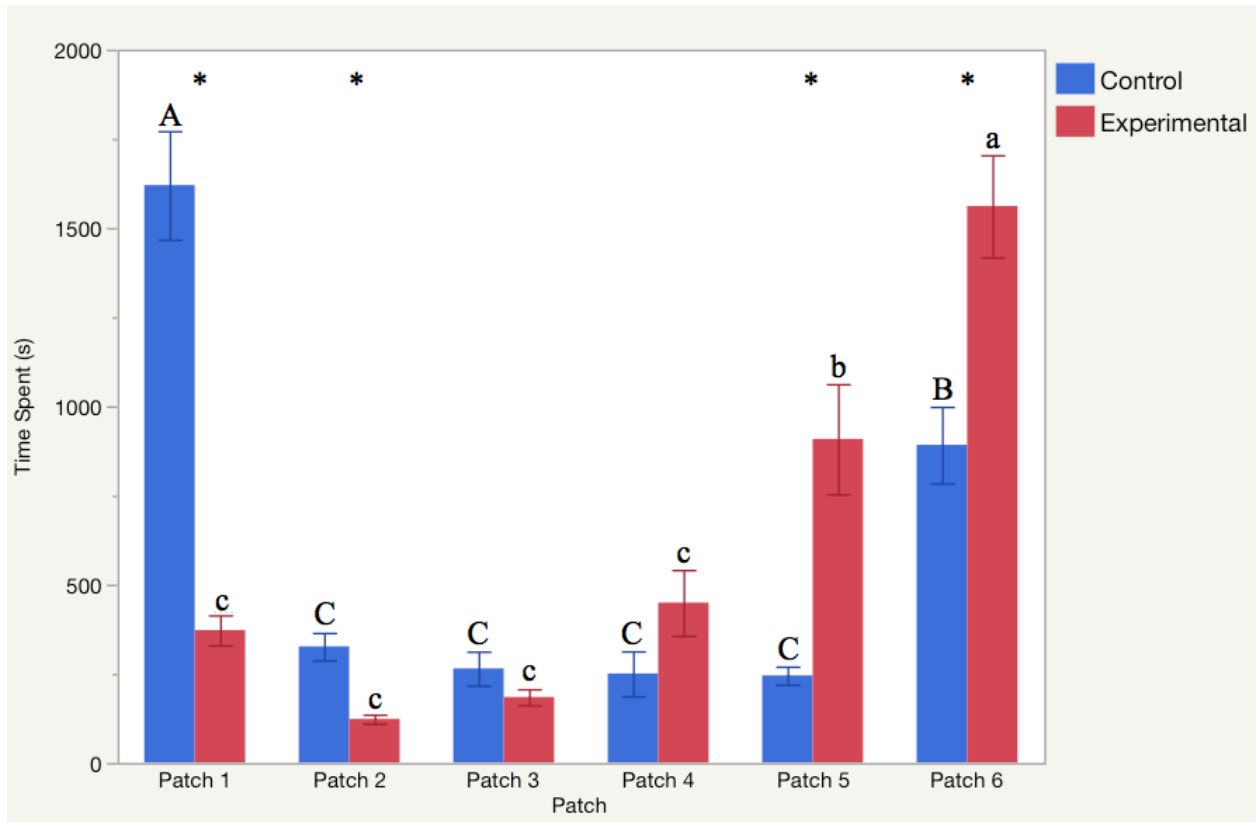


Figure 10. Salinity tolerance of red-backed salamanders during salinity gradient trials. The mean time red-backed salamanders spent on a patch in the control and experimental trials (mean  $\pm$  standard error). Control ANOVA:  $F_{5, 306} = 43.4779$ ,  $P < 0.0001$ . Experimental ANOVA:  $F_{5, 306} = 32.3663$ ,  $P < 0.0001$ ). Patches 1 through 6 consisted of 0 M NaCl in the control trials and 0.25, 0.2, 0.15, 0.1, 0.05, and 0 M NaCl in the experimental trials, respectively. Capitalized letters represent significant differences among control patches and lowercased letters represent significant differences among experimental patches (Tukey's HSD tests). Asterisks denote significant differences between control and experimental patches (sequential Bonferroni technique).  $N = 52$ .

Patch	<i>P</i> value	Comparison	Adjusted Significance Level	Significance
Patch 1	1.2748E-09	<	0.0083	Significant
Patch 2	6.9953E-07	<	0.0100	Significant
Patch 6	0.0001	<	0.0100	Significant
Patch 5	0.0002	<	0.0100	Significant
Patch 4	0.0716	>	0.0100	Not significant
Patch 3	0.1010	>	0.0100	Not significant

Table 1: Sequential Bonferonni technique among experimental vs. control mean patch

comparisons. Any *P* value greater than the adjusted significance level was classified as not significant. All patches in the control trials contained 0 M NaCl, while Patches 1 through 6 in the experimental trials contained 0.25, 0.2, 0.15, 0.1, 0.05, and 0 M NaCl, respectively.

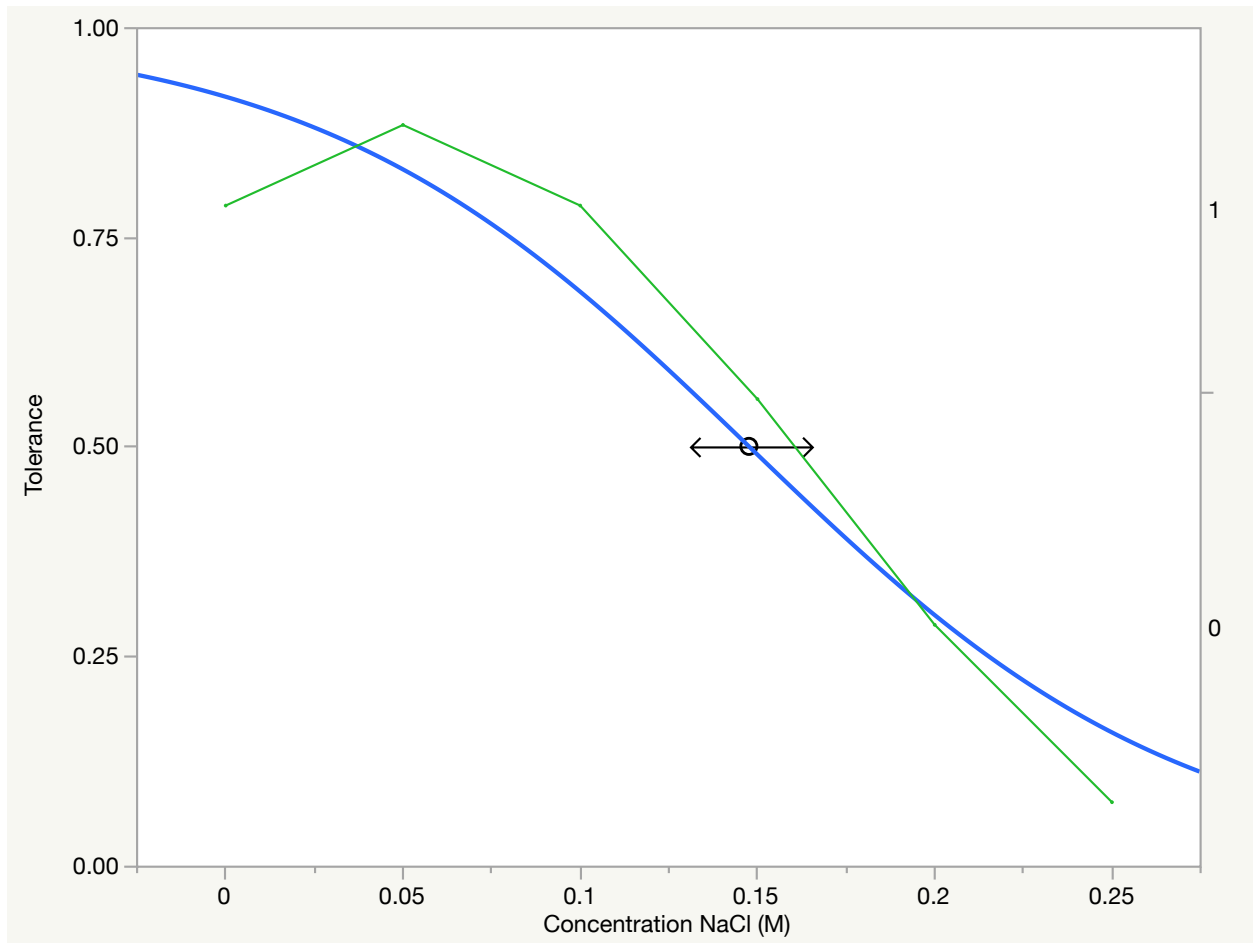


Figure 11. Nominal logistic regression curve of tolerance over salt concentration during salinity gradient trials. Salamanders decrease in tolerance as salinity intensity increases ( $\chi^2 = 104.4962$ ,  $P < 0.0001$ ).  $EC_{50} = 0.148$  M NaCl (95% C.I. = 0.131-0.166)

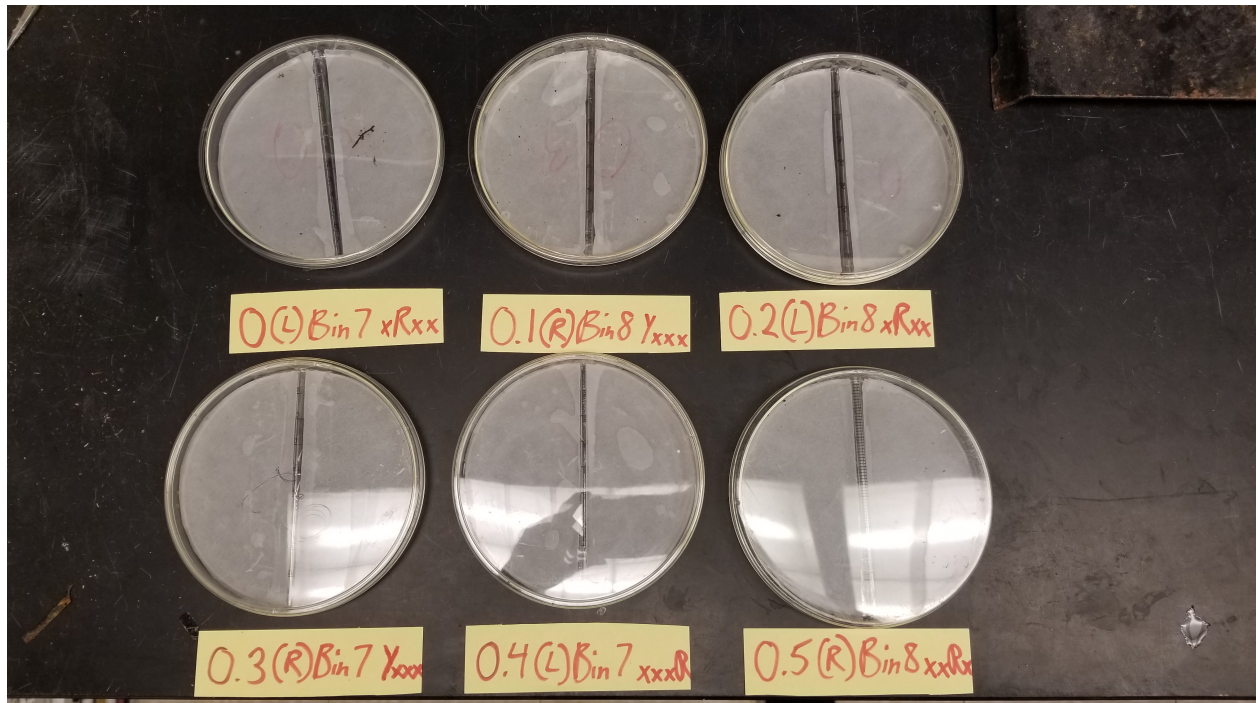


Figure 12: Salamander binary choice trial arrangement. Six 15-cm diameter petri dishes with two sides, control and experimental, divided by a traversable barrier. The filter papers on the control side contained 0 M NaCl, while experimental filter papers contained varied concentrations of either 0, 0.1, 0.2, 0.3, 0.4, or 0.5 M NaCl.

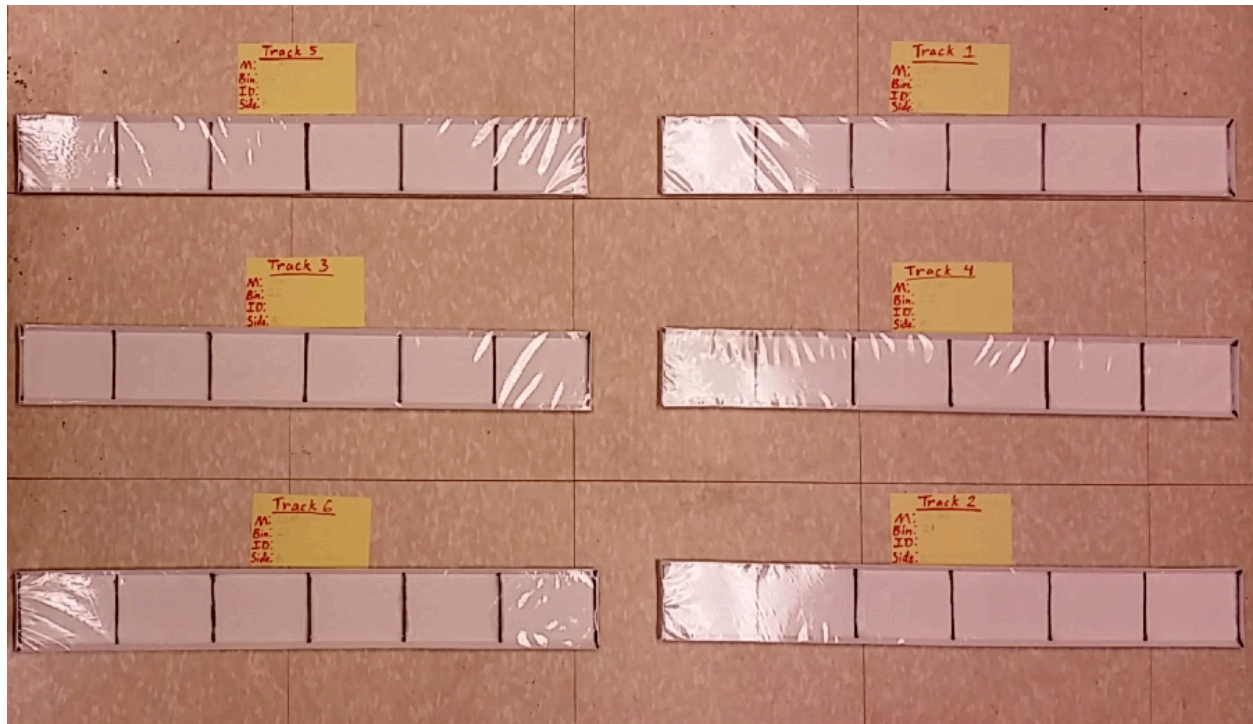


Figure 13: Salamander salinity gradient trial arrangement. Six tracks (91.4 cm long x 7.0 cm wide x 1.2 cm tall) had 5 traversable barriers and 6 15.2-cm x 7.0-cm segments with filter paper in each one. All track segments contained 0 M NaCl for the control trials. For the experimental trials, each track's segments sequentially contained 0.25, 0.2, 0.15, 0.1, 0.05 and 0 M NaCl.

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